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The type 1 copper site of pseudoazurin: Axial *and* rhombic

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ABSTRACT

We report on a high-frequency electron-paramagnetic-resonance study of the type 1 copper site of pseudoazurin. The spectra fully resolve the contribution of a nearly axial spectrum besides the rhombic spectrum, which unequivocally proves the existence of two conformations of the copper site. Pseudoazurins have been considered from *Achromobacter cycloclastes* including eight mutants and from *Alcaligenes faecalis*. The two conformations are virtually the same for all pseudoazurins, but the rhombic/axial population varies largely, between 91/9 and 33/67. These observations are discussed in relation to optical absorption spectra and X-ray diffraction structures. A similar observation for fern plastocyanin from *Dryopteris crassirhizoma* suggests that dual conformations of type 1 copper sites are more common.

1. Introduction

Since its discovery, the structure of the type 1 copper site in proteins and enzymes is the subject of extensive spectroscopic and quantum-chemical studies. The ligation of copper in type 1 sites by two histidines and a cysteine, completed by a weaker fourth ligand, which is most commonly a methionine, is best described as distorted tetrahedral, distinctly different from the square-planar copper coordination for inorganic copper(II) complexes. Initially, the restraint by the protein surrounding was considered to cause the difference [1,2], but quantum-chemical calculations on truncated model sites revealed that the non-planar geometry corresponds to an energy minimum already in vacuum [3,4]. Variation in the structure of the type 1 site from protein to protein derives from the interplay between the copper ligands and the surrounding protein matrix, i.e., the outer-sphere coordination [5].

The specific geometry of the type 1 sites goes hand in hand with a characteristic electronic structure. This is reflected in a relatively intense band around 600 nm in the visible absorption spectrum and a small copper hyperfine interaction in the low-field part of the EPR spectrum [6]. More specifically, type 1 sites are distinguished into two groups on the basis of the extent of rhombicity of the g tensor: "axial" sites as in e.g. azurin, plastocyanin, and amicyanin, and "rhombic" sites as in e.g. cucumber basic protein, rusticyanin, and pseudoazurin. The absorption spectrum shows in addition to the band at 600 nm a band around 450 nm, which is weak for the "axial" sites and stronger for the "rhombic" sites [7].

Extensive spectroscopic studies on proteins carrying the type 1 copper site showed the special nature of the copper–sulfur(Cys) interaction [8–12]. Circular dichroism and magnetic circular dichroism served to assign the ligand-field and charge-transfer transitions in the absorption spectra. Sulfur K-edge X-ray absorption, resonance Raman spectroscopy and EPR spectroscopy showed the high covalency of the copper–sulfur(Cys) bond. With reference to these observations, quantum-chemical calculations of increasing sophistication (SCF-Xα-scattered wave [8,13], density functional theory (DFT) and ab initio CASPT2 [4,14]) on model sites revealed the character of the singly occupied molecular orbital in the electronic ground state of type 1 copper proteins. This orbital is anti-bonding between copper and the cysteine sulfur, and the anti-bond is of pure π character for the axial sites and of mixed σ–π character for the rhombic sites. The former case corresponds to a distorted trigonal structure, the latter to a distorted tetrahedral/tetragonal structure [9,14]. In this way, the calculations on the truncated models provided insight into the essential features of the (electronic) structure of the family of type 1 copper sites in proteins and enzymes, and related the electronic properties of a specific site to its geometric structure.

In recent years, a number of observations challenged the picture of a unique relation between a protein/enzyme and the structure of its copper site. The occurrence of multiple structures of the type 1 site in one protein has been reported. The absorption spectrum of a mutant of amicyanin from *Paracoccus versutus* with a pseudoazurin loop

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showed a relative increase of the 450 nm absorption as compared to the 600 nm absorption with decreasing temperature [15], which was described in terms of the change of the relative population of two ground states corresponding to local energy minima [16]. For nitrite reductase a thermodynamic equilibrium was reported between a blue and a green type 1 copper site [17,18]. A particular intriguing case concerns pseudoazurin. For wild-type and mutants of pseudoazurin from *Achromobacter cycloclastes*, the ratio of the absorption intensities was found to vary with temperature and an axial contribution to the rhombic EPR spectrum was noticed [19,20]. These observations were interpreted in terms of two conformers. In contrast, a variable temperature spectroscopic study on pseudoazurin from *Paracoccus pantotrophus* indicated that a single species is present at all temperatures [21].

On the basis of optical studies it remains difficult to distinguish between the presence of distinct structures and gradual structural changes, because all type 1 copper sites absorb at 450 nm and at 600 nm and the resonance Raman spectra largely overlap. On the other hand, studies by EPR can discriminate between different structures as these are characterized by different g tensors. Contributions of an axial component to rhombic spectra have indeed been recognized for pseudoazurins from *Achromobacter cycloclastes* at the low-field edge of 9 GHz continuous-wave EPR spectra [19,20]. At this low microwave frequency the spectrum was not fully resolved. Here we show that 275 GHz EPR spectroscopy provides the g resolution necessary to unequivocally distinguish distinct contributions.

We have studied the 275 GHz continuous-wave EPR spectra of pseudoazurin from *Achromobacter cycloclastes* and eight mutants. Fig. 1 shows the structure of this pseudoazurin. The ligands of copper, His40, His81, Cys78, and Met86, are conserved for all systems studied. The mutations concern the residues Met16 and Thr36 in the outer coordination sphere. The Met16, which is in interaction with the neighboring copper ligand His81, has been replaced by amino acids carrying aliphatic or aromatic side chains [19,20]. The Thr36 is adjacent and hydrogen-bonded to His6, and the (de)protonation of this histidine is known to affect the structural properties of pseudoazurin [22]. This threonine is replaced by the positively charged lysine to compare the effect with that of protonation of His6. For comparison, we have included pseudoazurin from *Alcaligenes faecalis* [23] and plastocyanin from *Dryopteris crassirhizoma* [24]. The enhanced resolution at 275 GHz, as compared to the standard microwave frequency of 9 GHz, has enabled the detailed quantitative analysis of the EPR spectra. For all proteins studied, the spectra are found to be the weighted sum of an axial and a rhombic spectrum. The axial and rhombic spectra are essentially the same for all pseudoazurins, but the weights vary strongly. We combine this result with a further analysis of the visible absorption spectra, and discuss the consequences of our observations for the description of the type 1 copper site in general.

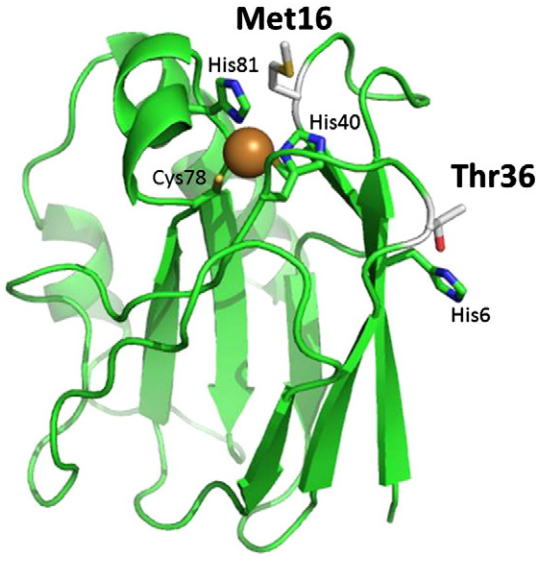

Fig. 1. Structure of pseudoazurin from *Achromobacter cycloclastes*.

2. Materials and methods

Wild-type pseudoazurin was isolated from *Achromobacter cycloclastes* [19] and *Alcaligenes faecalis* [25]. Apart from the wild-type protein from *Achromobacter cycloclastes* eight point mutants were purified following the method described in reference [19]. The protein concentration was determined by the BCA protein assay kit (Pierce). The copper content was determined for each protein with an inductively coupled plasma atomic emission spectrophotometer; a 1:1 concentration ratio of copper ion to protein was confirmed in each case. The molar extinction coefficients of the variant proteins were obtained on the basis of the copper ion concentration. Azurin was isolated from *Pseudomonas aeruginosa*, plastocyanin from *Dryopteris crassirhizoma*. The proteins were dissolved in a 20 mM potassium phosphate buffer and were concentrated using 3kD Amicon filters.

Continuous wave (cw) EPR spectra at 9 GHz were recorded at 80 K with a Bruker E680 ElexSys spectrometer at magnetic fields between 260 and 380 mT with a field modulation amplitude of 1 mT at 100 kHz. The protein solution was inserted into a quartz tube with an outer diameter of 4 mm and an inner diameter of 3 mm. For these measurements the samples were concentrated to 1 mM. The EPR spectra at 275 GHz were recorded at 15 K, using a homebuilt spectrometer operating at 275.7 GHz [26,27]. Field modulation amplitude was 1.4 mT at a frequency of a few hundred Hz. The protein solution, in 20 mM phosphate buffer pH7, was inserted into a quartz tube with an outer diameter of 250 μm and an inner diameter of 150 μm. Typically the protein concentration of the samples was 10 mM for the spectra shown in the figures. Similar, be it noisier, spectra were obtained for concentrations of 1 mM.

Electronic absorption spectra were recorded with a Shimadzu UV-2101PC spectro-photometer. Structural graphics and analyses were performed with the UCSF Chimera package using data from the RSCB Protein Data Bank.

3. Results

Continuous-wave EPR spectra at 275 GHz and 9 GHz have been measured of two wild-type pseudoazurins, and of six single mutants and two double mutants. Fig. 2 shows the spectra of wild-type pseudoazurin from *Achromobacter cycloclastes* at 275 GHz and at 9 GHz, and the spectrum of azurin from *Pseudomonas aeruginosa* at 275 GHz. The spectrum at 9 GHz of pseudoazurin (Fig. 2a) shows the known rhombic character of the spectrum for this protein. At 275 GHz (Fig. 2b), the anisotropy of the rhombic g tensor is completely resolved, with the g_x transition at 9.7780 T ($g = 2.015$), the g_y transition at 9.5080 T ($g = 2.072$), and the g_z transition at 8.9260 T ($g = 2.207$). Additional transitions are clearly visible at 9.6730 T ($g = 2.036$), 9.6040 T ($g = 2.051$) and 8.8140 T ($g = 2.235$), and the comparison with the 275 GHz spectrum of the axial copper site of azurin (Fig. 2c) suggests that these transitions derive from an extra axial component. (Note that we refer to this component still as "axial", although the EPR spectrum at 275 GHz clearly resolves the small rhombicity of the g-tensor of this component.) This second component is visible as well in the 9 GHz spectrum, be it only as a small band at the low-field side of the copper-hyperfine bands at g_z. At 275 GHz the copper-hyperfine interaction is not resolved, but hidden in the inhomogeneously broadened bands.

Fig. 3 shows three EPR spectra at 275 GHz, which are representative of the spectra for all pseudoazurins that we studied. (For the whole set of spectra at 9 GHz and at 275 GHz, see Figs. S1a and S2a of the supplementary material.) The spectra are the sum of two components, where